

Attorney Docket No.: PTQ-0027  
Inventors: Van Eyk et al.  
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Figure 1 is a graph showing the effect of reperfusion on the isometric force/pCa relation of Triton X-100 skinned cardiac muscle fiber bundles obtained from isolated rat hearts that experienced increasing durations of ischemia. Isometric force was measured as a function of increasing calcium concentrations for each skinned trabecula muscle bundle obtained from rats which had undergone 15 min of equilibrium followed by: 45 min of perfusion (control n=4,○), 15 min ischemia (n=4,△), 15 min ischemia followed by 45 min of reperfusion (n=4,▽), 60 min of ischemia (n=4,□) or 60 min ischemia followed by 45 min reperfusion (n=6,◇). Force is plotted as the percent of either the maximum force produced by the control skinned muscle fiber bundles ( $F_{\text{max}} = \text{force of fiber} / \text{maximum calcium-dependent force of control fiber bundle} (100\%)$ , panel A) or as the relative force (maximum calcium-dependent force of the fiber bundle = 100%, panel B) with respect to changing calcium concentration. The experimental protocols of the Langendorf perfusion and skinned muscle fiber bundle analysis are described below. Data are presented as  $\pm$ SEM for skinned fibers obtained from 4 to 6 rat hearts for each experimental condition. Where the error bars are not shown, the standard error is small and lies within the symbol.

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The paragraph, beginning on line 5, of page 6 has been amended as follows:

D<sup>2</sup>  
Figure 4 shows the results of an SDS-PAGE analysis of skinned left ventricle tissue samples from isolated rat hearts. Tissue samples obtained from hearts which experienced 15 min equilibration followed by either 45 min perfusion (control, 1), 15 min ischemia followed by 45 min reperfusion (i.e., 15/45; 2), 60 min ischemia (3) or 60 min ischemia followed by 45 minutes reperfusion (i.e., 60/45; 4) were skinned in 50% glycerol prior to being prepared for SDS-PAGE analysis. Panel A shows the coomassie blue stain of the 12.5% crosslinked gel. Panels B-F show corresponding western blots using anti- $\alpha$ -actinin (panel B), anti-TnI peptide residues 136 to 148 (MAb E2, panel C), anti-TnT (panel D), anti-TM (panel E), and anti-MLC1 (panel F) antibodies. Panel G shows the western blot of a 10% SDS-PAGE of control tissue and tissue obtained from rats which experienced 60 min ischemia (3). The western blot was probed with anti- $\alpha$ -actinin antibody. Modification products are indicated by arrows.

The paragraph, beginning at line 1, of page 7 has been amended as follows:

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D<sup>3</sup>  
Figure 6 shows the results of an SDS-PAGE analysis of isolated myofibrils from control and globally ischemic rat hearts. Left ventricular tissue samples obtained from isolated rat hearts were placed in saline in plastic bag for 60 min at either 4°C (control, 1) or 39°C (global ischemia, 2). Panel A shows the coomassie blue stain of the 12.5% crosslinked gel. Panels B to F show corresponding western blots using anti- $\alpha$ -actinin (panel B), anti-TnI peptide residues 136 to 148 (panel C), anti-TnT (panel D), and anti-MLC1 (panel E) antibodies. Modification products are indicated by arrows. The data reveal a loss of  $\alpha$ -actinin in the global ischemic myofibrils and degradation of TnI and MLC1, respectively.

The paragraph, beginning on line 10, of page 30, has been amended as follows:

D<sup>4</sup>  
Results of the SDS-PAGE analysis and subsequent western blots of rat heart reperfusion effluent are shown in Figure 3, and of tissue from global ischemic rat hearts in Figure 6, wherein MLC1 modification product is identified by an arrow (Figure 6E). Figure 8 and Figure 10C and 10D show that complexes are formed from fragments of TnI, TnT, and TnC. Figure 4 shows the SDS-PAGE analysis and subsequent western blots of rat skinned ventricular

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D4  
Cont  
tissue, wherein TnI modification products can be seen (Figure 4C, arrow). Note that  $\alpha$ -actinin was lost (Figure 4B) with mild ischemia, and  $\alpha$ -actinin degradation (Figure 4G) appeared with more severe ischemia.

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The paragraph, beginning at line 23, of page 31, has been amended as follows:

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Sub 32  
D5  
To identify the site of modification in troponin I, specific antibodies to the amino- and carboxyl-termini of troponin I were used to find out which antibodies bind to the different modification products. The antibodies MAb 10F2 (recognizes residues 188 to 199) and MAb 3350 (2F6.6) (recognizes residues 28 to 54) were used (Van Eyk et al. 1998, *Circ. Res.* 82:261-71). The various modification products were run on either a 12% SDS-PAGE or 10% T-PAGE (described in Schagger et al. 1987, *Analytical Biochemistry*, 166:368-79). The proteins were transferred to nitrocellulose using a 10 mM CAPS buffer pH 11.0 for 16 h at 27 V (described in Towbin et al. 1979, *PNAS* 76:4350-54). The carboxyl-terminus is usually the first to be clipped (Figure 7), yielding residues 1 to 193 (Figure 9), but in addition there are further modifications occurring at the amino-terminus with more severe

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5  
Dm ischemia (Figure 8A). Further TnI degradation products were identified as listed in Table 4.

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The paragraph, beginning at line 13, of page 38, has been amended as follows:

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D6 All tissue samples were excised and quickly washed in cold (4°C) saline before being frozen in liquid N<sub>2</sub> and stored at -70°C until prepared for SDS-PAGE analysis. Frozen tissue samples were homogenized in 25 mM Tris, pH 7.5, plus a cocktail of protease inhibitors (50 M phenylmethylsulfonyl fluoride, 3.6 M leupeptin, 2.1 M pepstatin A, and 10 mM EDTA). The protein contents of the homogenates were determined using Lowry assay. Protein samples were then prepared in Laemmli buffer (1% (w/v) SDS, 2.5 mM Tris, pH 6.5, 10% (w/v) sucrose, 0.025% (w/v) bromophenol blue) and 1 mM dithiothreitol at a total protein concentration of 1 mg/ml before being stored at -20°C for later SDS-PAGE and western immunoblot analysis.

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The comments following Table 3 have been amended as follows:

- \* The ischemia/reperfusion-induced modified TnI products observed in urea T-PAGE separated left ventricular tissue which underwent either 0/45, 15/45/ or 60/45 (Figure 8) were quantified from 8I-7

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MAB Western blots (Figure 8A). The quantity of each TnI component was determined as a percentage of the total TnI (intact and modified) present in each tissue sample. Only those products which are positively identified in Table 4 are included here, identified by their apparent molecular weight (Figure 8A).

† The ischemia/reperfusion-induced modified TnI products observed from 8I-7 MAB affinity chromatography of 60/45 left ventricular tissue (Figure 10) were quantified from 8I-7MAB Western blots (Figure 10B), and the amount of each TnI component determined as a percentage of the total in each sample.

‡ Control tissue, which experienced no ischemic episode, but 45 minutes of reperfusion.

§ The quantity of the two TnI-containing covalent complexes combined.

// Quantities less than 2% of total TnI could not be accurately determined.

The comments following Table 4 have been amended as follows:

\* TnI products identified by their apparent molecular weights (Figure 8A).

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† Immunological analysis (Western blots, Figures 8A, 10C) of protein products bound to Mabs: strong (+), weak ( $\pm$ ), or no binding (-).

‡ Electrophoretic mobility in alkaline urea PAGE (Figure 10): mobile (+, TnC containing), non-mobile (-, not containing TnC).

§ The amino acid sequences(s) of proteins which are the theoretical best match to the observed masses.

// Best match to the observed masses was determined by calculating the mass of rcTnI, rcTnT, and mouse cTnC, sequentially clipped from the N- and C-termini using the PeptideMass tool from the Swiss Institute for Bioinformatics website.

¶ The source of the TnI products indicates the peak from RP-HPLC analyzed 8I-7 affinity column fractions of 60/45 tissue (Fig 4).

# Mass determined by electrospray mass spectrometry.

\*\* Mass determined by matrix assisted laser desorption/ionization mass spectrometry.

†† The difference between the observed and theoretical masses is equal to that of a sodium ion (MW 35 Da), which is commonly found associated with mass spectrometrically analyzed proteins (as a result of the ionization process).